*Original Article*

# A Randomized Trial of Partial Versus Complete Versus Not Using Laser-Assisted Hatching in Vitrified-Warmed Cleavage Embryo Transfer: A Preliminary Report

Usanee Sanmee MD[1], Waraporn Piromlertamorn BSc[1], Teraporn Vutyavanich MD[1]

[1] Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

***Background***: Currently, the effect of laser-assisted hatching (LAH) on the outcome of cryopreserved embryo remains controversial and unclear, especially on the cryopreserved embryos using a novel vitrification method.

***Objective***: To compare the pregnancy outcomes of vitrified-warmed cleavage stage embryos transfer using LAH breaching or LAH thinning versus those not using LAH.

***Materials and Methods***: Sixty patients with vitrified-warmed cleavage embryo transfer were randomly assigned to a control group without LAH treatment, LAH-breeching group, and LAH-thinning group. The outcome measurements were clinical pregnancy rate, implantation rate, and live birth rate.

***Results***: The clinical pregnancy rate (35% versus 20% versus 25%) and implantation rate (17.3% versus 11.5% versus 11.3%) were lower in both LAH-breaching and LAH-thinning group than the control group, but not statistically significant (p>0.05). The live birth rate (30% versus 5% versus 5%) was significantly lower in both the LAH-breaching and LAH-thinning group than the control group (p=0.026).

***Conclusion***: LAH regardless of breaching or thinning methods significantly decreases live birth rate in vitrified-warmed cleavage-stage embryo transfer.

***Keywords***: Laser-assisted hatching, Vitrified-warmed, Cleavage embryo

Received 8 June 2020 | Revised 6 August 2020 | Accepted 6 August 2020

**J Med Assoc Thai 2021;104(1):18-23**

**Website:** http://www.jmatonline.com

Assisted hatching (AH) is one method believed to increase the pregnancy rate in assisted reproductive technologies (ART) treatment. This technique will solve the problem of zona pellucida (ZP) hardening from suboptimal culture conditions during in vitro culture and after cryopreservation procedure[1,2]. Failure to hatch of an embryo can prevent implantation. Therefore, an artificial manipulation to help embryos exit the ZP will increase the capability of the embryo to implant. Although there are several AH techniques, laser AH is the most widely used in the clinical setting because it is easy to use, fast, accurate, and safe. Clinical studies indicating that the success rates following the use of laser-assisted hatching (LAH) in different ART programs remain unclear and controversial[3,4]. Currently, there is insufficient evidence to recommend AH in all cryopreserved embryo transfer cycles. A variety of study results may be from numerous factors, including 1) zona thickness after AH, to create a full-thickness hole in ZP, which called zona breaching[5,6] or to thin an extensive area of the ZP with kept intact the inner zona, which called zona thinning[7-10], 2) size of zona opening[11], 3) the embryo stage while performed AH, cleavage, or blastocyst stage[12], and 4) embryo cryopreservation technique, slow freezing[5,9,13], or vitrification, the different methods will affect the ZP differently. Vitrification is employed more and more often as it has less chilling injuries due to no ice crystal formation[14], no need for an expensive instrument, minimal set up time is required, and survival rate after warming is comparable to or even higher than that in the slow freezing method[15]. The present study was set up to compare the pregnancy outcomes of vitrified-warmed cleavage stage embryos transfer

**Correspondence to:**

Sanmee U.

Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.

**Phone**: +66-53-935552, **Fax**: +66-53-946112 ext. 3

**Email**: usanee.s@cmu.ac.th

**How to cite this article:**

Sanmee U, Piromlertamorn W, Vutyavanich T. A Randomized Trial of Partial Versus Complete Versus Not Using Laser-Assisted Hatching in Vitrified-Warmed Cleavage Embryo Transfer: A Preliminary Report. J Med Assoc Thai 2021;104:18-23.

doi.org/10.35755/jmedassocthai.2021.01.11515

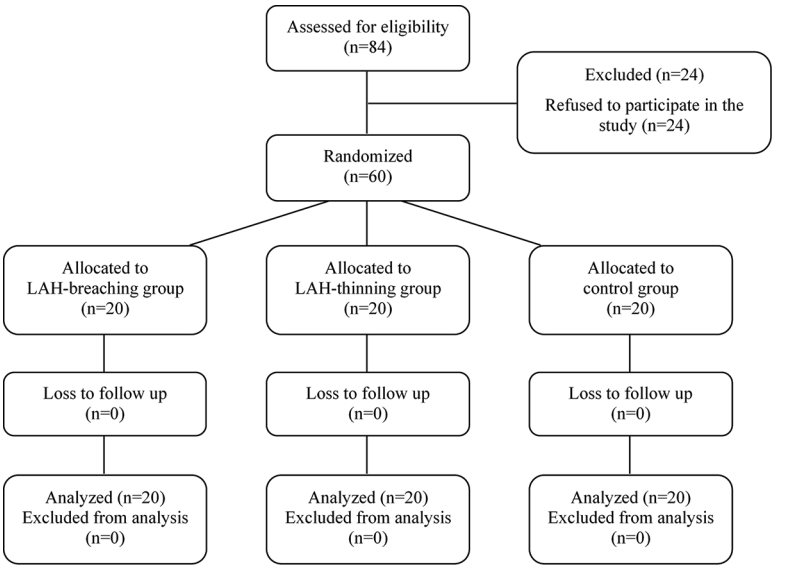


**Figure 1.** Flow diagram of patients through each stage of the study.

using LAH-breaching or LAH-thinning versus those not using LAH.

## Materials and Methods

### Patients

The present study was a randomized, controlled trial, that began after approval was obtained from the Institutional Review Board of the Faculty of Medicine, Chiang Mai University. The study was conducted at the reproductive medicine unit of Maharaj Nakorn Chiang Mai Hospital. On the day of embryo transfer, patients who met the following inclusion criteria were invited to participate 1) ages between 20 to 42 years, 2) had cleavage stage embryos cryopreserved by vitrification method and post warmed resulting in 100% intact blastomere, 3) the embryos were transferred in the cleavage stage, and 4) had an endometrial thickness of 8 mm or more on the day of embryo transfer. Patients with uterine fibroid, hydrosalpinx, or endometrioma were excluded. Patients were randomized into a control group without LAH treatment, LAH-breaching group to create the full thickness 30 µm hole on ZP before embryo transfer, and LAH-thinning group to thin 90% of ZP thickness one quarter before embryo transfer. The randomization technique was performed by a computer-generated random numbers sealed in envelops. All patients received an embryo transfer by the same doctor. After embryo transfer, luteal-phase support was achieved for two weeks. Serum beta-human chorionic gonadotropin (β-hCG) was measured two weeks later, and clinical pregnancy was confirmed when a fetal heartbeat was identified via ultrasound five weeks after transfer. If pregnancy was achieved, the luteal support was continued until 12 weeks of gestation. The trial design is shown in Figure 1.

### Vitrification and warming protocol

The embryos were cryopreserved by in house vitrification methods. The freezing program was performed at room temperature as follows, the embryos were transferred into 500 µl of 10% human serum albumin (HSA) in phosphate buffer saline (PBS) for five minutes; then moved to vitrification solution 1 (VS1) containing 10% ethylene glycol (EG) with 10% HSA in PBS for seven minutes, then moved to vitrification solution 2 (VS2) containing 35% EG with 10% HSA in PBS with 1M sucrose and the embryos were placed on the hemi-straw in a small drop within one minute. The hemi-straw was inserted into an outer straw in the aluminum block, which was pre-cooled in liquid nitrogen for 20 minutes before use.

The three warming solutions (WS1, WS2, and WS3), contained 1M, 0.5M, and 0M sucrose, respectively, with 10% HSA in PBS. They were warmed to 37°C before use. Embryo warming was performed on the morning of transfer day. The warming procedure was as follows, the outer straw was removed under the liquid nitrogen, and the tip of the hemi-straw was quickly submerged into 500

**Table 1.** Patient characteristics

| Parameter | Untreated zona pellucida; n (%) | Laser zona pellucida breeching; n (%) | Laser zona pellucida thinning; n (%) | p-value |
|---|---|---|---|---|
| No. of cycle | 20 | 20 | 20 | |
| IVF cycles | 9/20 (45) | 10/20 (50) | 7/20 (35) | 0.897 |
| ICSI cycles | 11/20 (55) | 10/20 (50) | 13/20 (65) | 0.865 |
| Female age (years) at; mean±SD | | | | |
| Vitrification | 33.21±3.84 | 33.70±4.21 | 34.75±3.86 | 0.470 |
| Warming | 33.42±3.76 | 34.80±4.40 | 35.00±3.76 | 0.413 |
| Primary infertility | 15/20 (75) | 16/20 (80) | 16/20 (80) | 0.889 |
| Secondary infertility | 5/20 (25) | 4/20 (20) | 4/20 (20) | 0.874 |
| Duration of infertility (years); mean±SD | 6.06±4.48 | 4.60±2.77 | 4.88±3.74 | 0.505 |
| Cause of infertility | | | | |
| Male factor | 8/20 (40) | 8/20 (40) | 10/20 (50) | 0.772 |
| Tubal factor | 2/20 (10) | 2/20 (10) | 2/20 (10) | 0.374 |
| Unexplained | 10/20 (50) | 10/20 (50) | 8/20 (40) | 0.774 |
| Previous ART cycles | 3/20 (15) | 4/20 (20) | 0/20 (0) | 0.099 |
| Previous fresh transfer | 15/20 (75) | 15/20 (75) | 14/20 (70) | 0.618 |
| Previous frozen-thawed embryo transfer | 4/20 (20) | 5/20 (25) | 4/20 (20) | 0.926 |

IVF=in vitro fertilization; ICSI=intracytoplasmic sperm injection; ART=assisted reproductive technology; SD=standard deviation

There were no statistically significant differences between the three groups

µl of WS1for five minutes. Then, the embryos were moved to WS2 and WS3 for five minutes each, then washed and cultured. Embryos were cultured in a tri-gas incubator at 37°C in an atmosphere of 6% $CO_2$, 5% $O_2$, and 89% $N_2$.

### Embryo grading

The cleavage-stage embryo was scored using Zieba et al criteria[16]. The embryos with 6 to 10 cells with even blastomeres and less than 10% fragmentation are defined as grade A, embryos with uneven blastomeres and 10% to 20% fragmentation are defined as grade B, embryos with 20% to 50% fragmentation are defined as grade C, and embryos with more than 50% fragmentation are defined as grade D.

### Laser-assisted hatching procedure

The LAH was performed immediately before the embryo transfer using non-contact diode laser at a wavelength of 1.48 µm of the ZILOS-tk Zone Infrared Laser Optical System (Hamilton Thorne Instruments Biosciences, Beverly, Massachusetts). The power routinely available at the image plane of the objective was 47 mW. The embryos were treated directly in their original culture medium in a culture dish, which was placed onto the inverted microscope (Olympus, Tokyo, Japan) equipped with a heated stage (Kitazato, Fujinomiya, Japan). The laser beam was fired at the ZP to create a full-thickness opening of 30 µm in the LAH-breaching group. To minimize the detrimental effect, the targeted laser located at the ZP that has a clear space between the inner membrane of the ZP and the blastomeres to ensure that the blastomeres were not touched by the laser. For the LAH-thinning group, the same setting was used to fire in a consecutive area along the periphery of ZP to leave the thin rim of about 10% of the original thickness, covering an area of a quarter of the ZP circumference. The LAH procedure was carried out in approximately 30 seconds per embryo. After the LAH procedure, the embryos were moved to a transfer dish to proceed with the embryo transfer. All the LAH procedures were performed by the same embryologist.

### Statistical analysis

The SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. Qualitative variables were compared using the chi-square test. Continuous variables were performed using ANOVA when data distribution was normal or Kruskal Wallis test when normality could not be confirmed. A p-value of less than 0.05 was considered statistically significant.

## Results

Between September 2012 and October 2014, 60 patients that met the inclusion criteria were

**Table 2.** Laboratory and clinical outcomes of vitrified-warmed embryo transfers

| Parameter | Untreated zona pellucida; n (%) | Laser zona pellucida breeching; n (%) | Laser zona pellucida thinning; n (%) | p-value |
|---|---|---|---|---|
| No. of cycles | 20 | 20 | 20 | |
| Vitrified embryos per patient; mean±SD | 9.29±7.92 | 6.33±3.03 | 7.50±4.66 | 0.779 |
| Warmed embryos per patient; mean±SD | 2.84±0.50 | 3.05±0.60 | 3.30±1.42 | 0.599 |
| Survival rate per warmed embryo | 52/54 (96.3) | 52/56 (92.9) | 53/58 (91.4) | 0.566 |
| Morphology of embryo before transfer | | | | |
| Grade A | 28/52 (53.8) | 38/52 (73.1)a | 25/53 (47.2)a | 0.020 |
| Grade B | 20/52 (38.5) | 13/52 (25.0) | 24/53 (45.3) | 0.090 |
| Grade C | 3/52 (5.8) | 1/52 (1.9) | 4/53 (7.5) | 0.414 |
| Grade D | 1/52 (1.9) | 0/52 (0.0) | 0/53 (0.0) | 0.367 |
| Zona pellucida thickness (μm); mean±SD | 17.85±2.96 | 18.21±4.55 | 18.18±4.08 | 0.645 |
| No. of embryos transfer per patient; mean±SD | 2.63±0.50 | 2.60±0.50 | 2.65±0.50 | 0.948 |
| Clinical pregnancy rate per transfer cycle | 7/20 (35.0) | 4/20 (20.0) | 5/20 (25.0) | 0.564 |
| Implantation rate per embryos transfer | 9/52 (17.3) | 6/52 (11.5) | 6/52 (11.3) | 0.600 |
| Miscarriage rate | 1/20 (5.0) | 3/20 (15.0) | 4/20 (20.0) | 0.371 |
| Live birth rate per transfer cycle | 6/20 (30.0)b,c | 1/20 (5.0)b | 1/20 (5.0)c | 0.026 |

SD=standard deviation

Chi-square test: a p=0.019, b p=0.026, c p=0.026

enrolled in the present study and randomized into 20 patients per group. The patients' characteristics in each group are shown in Table 1. There were no statistically significant differences in age at vitrification (33.21±3.84 versus 33.70±4.21 versus 34.75±3.86), age at warming (33.42±3.76 versus 34.80±4.40 versus 35.00±3.76), primary or secondary infertility type, duration of infertility (6.06±4.48 versus 4.60±2.77 versus 4.88±3.74), cause of infertility, and number of the previous failed cycles between the three groups (p>0.05).

The laboratory and clinical outcomes of vitrified-warmed embryo transfer in each group are shown in Table 2. There was no difference in the number of warmed embryos per patient (2.84±0.05 versus 3.50±0.60 versus 3.30±1.42), the survival rate of the vitrified embryo after warming (96.3% versus 92.9% versus 91.4%), and the number of embryos transferred per patient (2.63±0.50 versus 2.60±0.50 versus 2.65±0.50) among the three groups (p>0.05). There was no embryo left to continue an embryo culture in all patient.

The clinical pregnancy rate (35% versus 20% versus 25%) and implantation rate (17.3% versus 11.5% versus 11.3%) were lower in both LAH-breaching and LAH-thinning group than in the control group, but not statistically significant (p>0.05). The miscarriage rate (5% versus 15% versus 20%) was higher in both LAH-breaching and LAH-thinning group than in the control group, but not statistically significant (p>0.05). The live birth rate (30% versus 5% versus 5%) was significantly lower in both the LAH-breaching and LAH-thinning group than in the control group (p=0.026, Table 2). Among the live births, there are four singletons and two dizygotic twins in the control group, one dizygotic twin in the LAH-breaching group and one dizygotic twin in LAH-thinning group. Among the newborns, no congenital malformation was found in both the control and LAH groups.

## Discussion

The ZP is a glycoprotein layer that surrounds the embryo to prevent blastomere loss and protect the embryo from the environment when it moves through the fallopian tube to the endometrial cavity. During the blastocyst stage, the embryo hatches from the ZP and begins the implantation process. Failure to hatch of the embryo results in a failed implantation. Therefore, aside from embryo quality, the hatching process is considered the key to successful implantation. The hatching process involves physical repeated expansion and collapse of the blastocyst together with enzymatic digestion from embryo and endometrium to thinning and rupture of the ZP[17]. Vitrified-warmed process and in vitro culture condition cause a hardening of the ZP, which interfere with the natural hatching mechanisms[18]. However, the effect of LAH on the outcome of the cryopreserved embryo remains unclear. Some studies

suggest that LAH has beneficial effects on pregnancy rate in cryopreserved embryos[9,11,19,20], others claim that it has no benefit[8,10,13] and interestingly some studies even show the negative impact of LAH on pregnancy rates[7]. Only a few studies were conducted on embryos following the vitrification method, and most of the data is from a slow freezing cryopreserved embryo. Nowadays slow freezing has been substituted by a novel vitrification method. Therefore, more data on the effect of LAH on vitified-warmed embryos are needed.

In the present study, with respect to the vitrified-warmed cleavage embryo transferred cycles, patients who underwent LAH in the present study regardless of breaching or thinning methods showed significantly lower live births rates (30% versus 5% versus 5%, $p<0.05$). The clinical pregnancy rate (35% versus 20% versus 25%) and implantation rate (17.3% versus 11.5% versus 11.3%) in the LAH groups regardless of breaching or thinning method were also lower than the control group, however, this difference was not significant ($p>0.05$). This finding showed a similar result with Valojerdi et al, which was conducted on vitrified-warmed cleavage stage embryos[7]. That study reported a significantly lower pregnancy rate (28.5% versus 43%, $p=0.002$) and implantation rate (11.2% versus 16.7%, $p=0.004$) in the LAH-thinning group compared to the control group.

The present study findings suggest that LAH contributes unfavorable clinical outcomes for vitrified-warmed cleavage stage embryos. This may be due to many factors such as the creation of the full thickness hole in the cleavage embryo instead of the blastocyst stage naturally will 1) interfere with a natural embryo expansion[21], 2) the blastomere will be lost through the hole before the compaction stage, 3) the hole size, 30 µm in the present study, may be too small resulting in complete hatching, and 4) early exposure of the cleavage embryo to the environment that might increase risk for immune system rejection and infection. Besides, a potential problem of LAH is the local heating caused by the laser beam power that may have adverse effects on the embryo viability, quality, and developmental potential[22]. Especially in the LAH-thinning group that uses several laser shots to thin a quarter of ZP, which needs precise and good skills for safety and rapid performance of the procedure. There was no evidence of increased chromosomal abnormalities or congenital malformations in children born after LAH[23]. The present study found no evidence of any harmful effect of LAH on newborns.

Studies have showed that LAH has been associated with an increased risk of monozygotic or multiple pregnancies[24]. The authors did not find any monozygotic twins in the present study, but the number of cases reported in the present study was too small to make a conclusion.

## Conclusion

LAH, regardless of breaching or thinning methods, significantly decreased the live birth rate in vitrified-warmed cleavage stage embryo transfer. Therefore, the authors suggested that its use must be carefully considered. However, the sample size is small, so, further larger sample is still needed.

## What is already known on this topic?

At present, the effect of LAH on the outcome of cryopreserved embryo remains controversial and unclear. Some studies showed beneficial effect of LAH on pregnancy rates, others suggested no benefit, and few studies showed the negative effects of LAH. Therefore, currently there is insufficient evidence to recommend LAH in all cryopreserved embryo transfer cycles.

## What this study adds?

This study investigated the effect of LAH on cryopreserved embryos using a novel vitrification method. Most of the current studies data came from cryopreserved embryos using slow-freezing method. Nowadays, most ART centers use a vitrification method to cryopreserve embryos. The authors compared the pregnancy outcomes of vitrified-warmed cleavage stage embryos transfer using two commonly used LAH methods, LAH- breaching or LAH-thinning, versus those not using LAH. The present study findings found that LAH regardless of breaching or thinning methods not only has no beneficial effect but also showed an unfavorable clinical outcome on live birth rates.

## Acknowledgement

This research was supported by the Faculty of Medicine Endowment Fund for Medical Research, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

## Conflicts of interest

The authors declare no conflict of interest.

## References

1. al-Nuaim LA, Jenkins JM. Assisted hatching in assisted

reproduction. BJOG 2002;109:856-62.
2. Carroll J, Depypere H, Matthews CD. Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. J Reprod Fertil 1990;90:547-53.
3. Sallam HN, Sadek SS, Agameya AF. Assisted hatching--a meta-analysis of randomized controlled trials. J Assist Reprod Genet 2003;20:332-42.
4. Seif MM, Edi-Osagie EC, Farquhar C, Hooper L, Blake D, McGinlay P. Assisted hatching on assisted conception (IVF & ICSI). Cochrane Database Syst Rev 2005;(4):CD001894.
5. Gabrielsen A, Agerholm I, Toft B, Hald F, Petersen K, Aagaard J, et al. Assisted hatching improves implantation rates on cryopreserved-thawed embryos. A randomized prospective study. Hum Reprod 2004;19:2258-62.
6. Check JH, Hoover L, Nazari A, O'Shaughnessy A, Summers D. The effect of assisted hatching on pregnancy rates after frozen embryo transfer. Fertil Steril 1996;65:254-7.
7. Valojerdi MR, Eftekhari-Yazdi P, Karimian L, Hassani F, Movaghar B. Effect of laser zona thinning on vitrified-warmed embryo transfer at the cleavage stage: a prospective, randomized study. Reprod Biomed Online 2010;20:234-42.
8. Petersen CG, Mauri AL, Baruffi RL, Oliveira JB, Felipe V, Massaro FC, et al. Laser-assisted hatching of cryopreserved-thawed embryos by thinning one quarter of the zona. Reprod Biomed Online 2006;13:668-75.
9. Balaban B, Urman B, Yakin K, Isiklar A. Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: a prospective randomized study. Hum Reprod 2006;21:2136-40.
10. Ng EH, Naveed F, Lau EY, Yeung WS, Chan CC, Tang OS, et al. A randomized double-blind controlled study of the efficacy of laser-assisted hatching on implantation and pregnancy rates of frozen-thawed embryo transfer at the cleavage stage. Hum Reprod 2005;20:979-85.
11. Hiraoka K, Fuchiwaki M, Hiraoka K, Horiuchi T, Murakami T, Kinutani M, et al. Effect of the size of zona pellucida opening by laser assisted hatching on clinical outcome of frozen cleaved embryos that were cultured to blastocyst after thawing in women with multiple implantation failures of embryo transfer: a retrospective study. J Assist Reprod Genet 2008;25:129-35.
12. Vanderzwalmen P, Bertin G, Debauche C, Standaert V, Bollen N, van Roosendaal E, et al. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum Reprod 2003;18:1504-11.
13. Ng EH, Lau EY, Yeung WS, Cheung TM, Tang OS, Ho PC. Randomized double-blind comparison of laser zona pellucida thinning and breaching in frozen-thawed embryo transfer at the cleavage stage. Fertil Steril 2008;89:1147-53.
14. Bagis H, Mercan HO, Cetin S, Sekmen S. The effect of equilibration time on survival and development rates of mouse pronuclear-stage embryos vitrified in solid surface (SSV) and convential straws: in vitro and in vivo evaluations. Mol Reprod Dev 2005;72:494-501.
15. AbdelHafez FF, Desai N, Abou-Setta AM, Falcone T, Goldfarb J. Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis. Reprod Biomed Online 2010;20:209-22.
16. Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Andersen AN. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Hum Reprod 1997;12:1545-9.
17. Hammadeh ME, Fischer-Hammadeh C, Ali KR. Assisted hatching in assisted reproduction: a state of the art. J Assist Reprod Genet 2011;28:119-28.
18. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, et al. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod 1990;5:7-13.
19. Ge HS, Zhou W, Zhang W, Lin JJ. Impact of assisted hatching on fresh and frozen-thawed embryo transfer cycles: a prospective, randomized study. Reprod Biomed Online 2008;16:589-96.
20. Valojerdi MR, Eftekhari-Yazdi P, Karimian L, Ashtiani SK. Effect of laser zona pellucida opening on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen-thawed embryos. Fertil Steril 2008;90:84-91.
21. Montag M, van der Ven H. Laser-assisted hatching in assisted reproduction. Croat Med J 1999;40:398-403.
22. Edwards JL, Hansen PJ. Differential responses of bovine oocytes and preimplantation embryos to heat shock. Mol Reprod Dev 1997;46:138-45.
23. Kanyó K, Konc J. A follow-up study of children born after diode laser assisted hatching. Eur J Obstet Gynecol Reprod Biol 2003;110:176-80.
24. Alikani M, Noyes N, Cohen J, Rosenwaks Z. Monozygotic twinning in the human is associated with the zona pellucida architecture. Hum Reprod 1994;9:1318-21.